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RESEARCH ARTICLE

Exploitation of the Ornithine Effect Enhances Characterization of Stapled and Cyclic Peptides

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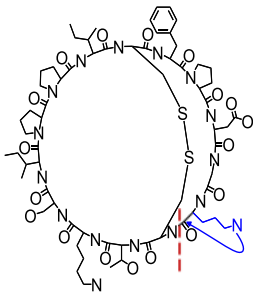

Abstract. A method to facilitate the characterization of stapled or cyclic peptides is reported via an arginine-selective derivatization strategy coupled with MS/MS analysis. Arginine residues are converted to ornithine residues through a deguanidination reaction that installs a highly selectively cleavable site in peptides. Upon activation by CID or UVPD, the ornithine residue cyclizes to promote cleavage of the adjacent amide bond. This Arg-specific process offers a unique strategy for site-selective ring opening of stapled and cyclic peptides. Upon activation of each derivatized peptide, site-specific backbone cleavage at the ornithine residue results in two complementary products: the lactam ring-containing portion of the peptide and the amine-containing portion. The deguanidination process not only provides a specific marker site that initiates fragmentation of the peptide but also offers a means to unlock the staple and differentiate isobaric stapled peptides.

Keywords: Cyclic peptide, Stapled peptide, Ornithine effect

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Introduction

Cyclic, stapled, and branched peptides constitute a unique and growing class of biomolecules with promise as therapeutics because of their biostability and resistance to proteolytic digestion in physiological environments [1–7]. In the context of therapeutics, peptide-based drug candidates display both advantages and disadvantages in comparison to their small molecule counterparts. Peptides, while frequently less toxic than small molecules when administered intravenously and exhibiting higher selectivity for specific biological functions, do not traverse cell membranes with the ease that small molecules do and are prone to proteolytic degradation [8, 9]. However, peptides that are protected from degradation via cyclization or stapling have been shown to exhibit high potency and low toxicity, resulting in more promising candidates for drug administration than their linear counterparts [3–7]. A myriad of nonlinear peptides are found naturally in plants, fungi, and bacteria as well as synthetic ones produced in the laboratory [10–18]. Valinomycin, for example, is a cyclic dodecadepsipeptide produced by *Streptomyces fulvissimus* with potent antibacterial properties while also acting as a potassium-selective ionophore [19, 20]. Enzyme inhibition is another pharmaceutical application that is associated with cyclic peptides, as shown by two examples of the Bowman-Birk class of protease inhibitors, Sunflower trypsin inhibitor-1 (SFTI-1) and *Momordica cochinchinensis* trypsin inhibitor-II (MCoTI-II) [21].

From an analytical standpoint, structural characterization of cyclic or stapled peptides is significantly more challenging than elucidation of linear peptides. The success of tandem mass spectrometry for sequencing peptides is based on production of predictable N-terminus and C-terminus fragment ions via cleavage of the peptide backbone. Cyclic peptides require cleavage of two backbone bonds to generate fragment ions, and the lack of natural N-terminal and C-terminal positions confounds an orderly mapping of the sequence of residues. Stapled peptides suffer from a similar pitfall in the region of the peptide containing the stapled (cyclized) portion and also typically contain hydrocarbon (non-peptide-like) linkers that

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constitute the staple. Several mass spectrometric techniques, including collision induced dissociation (CID) [20–25], MS^n methods [23, 25–27], electron capture dissociation (ECD) [28], and complexation strategies [29, 30], have emerged as the most valuable tools to assist in the characterization of the sequences of non-linear peptides.

In addition to the use of MS^n methods to facilitate the characterization of cyclic and stapled peptides, another synergistic strategy evolves from site-selective fragmentation processes that may be used to "anchor" a particular location in a molecule based on a site-specific cleavage. Once an anchor point is established, all other fragmentation pathways and the resulting product ions can be referenced to the anchor point. Highly selective or preferential bond-specific cleavages remain relatively rare occurrences upon activation of peptides and proteins. As one example, the proline effect is one of the few well-established site-specific cleavage upon activation, occurring N-terminal to proline residues due to the increased basicity of the N-alkylated amide bond as well as the increased steric hindrance about the residue [31, 32]. The proline-directed cleavage creates a readily recognized fragment ion in the MS/MS spectra of peptides. Along these lines, there has been renewed interest in the development of peptide derivatization strategies to install tags with labile bonds or ones with selectively cleavable groups in order to promote bond-selective cleavages [33–39]. Recently, the "ornithine effect" has been reported as a site-specific cleavage occurring C-terminal to ornithine residues [40, 41]. Upon collisional activation of an ornithine-containing peptide, the amine of the ornithine residue cyclizes via nucleophilic attack at the adjacent carbonyl group, resulting in a characteristic and preferential cleavage C-terminal to the carbonyl group. This phenomenon has been observed before, as lysine and many of its homologues have exhibited similar characteristics as a nucleophile previously [42–45].

The present study explores the utility of the ornithine effect to enhance the characterization of cyclic and stapled peptides. By exploiting the predictability of the ornithine effect, unique ions are generated that allow the differentiation of isomers. Both collision-based and photon-based activation methods are used to characterize the modified and unmodified peptides. The fragment ions were assigned using an in-house algorithm designed to systematically generate a list of every possible combination of bond cleavages (including cross-ring cleavages) and the associated masses of the fragments.

Experimental

Materials and Reagents

HPLC grade water and methanol used for sample preparation and dilution were purchased from EMD Millipore (Billerica, MA, USA) and formic acid was purchased from Fisher Scientific (Fairlawn, NJ, USA). Sunflower trypsin inhibitor-1 (SFTI-1) was provided by Bristol-Myers Squibb (Princeton, NJ, USA). Dithiothreitol (DTT) and iodoacetamide (IAM) used for reduction and alkylation of disulfide bonds were purchased from Sigma Aldrich (St. Louis, MO, USA). Proteomics-grade trypsin was obtained from Promega (Madison, WI, USA). The stapled peptides were synthesized as described in the Supplemental Information.

Synthesis and Purification of Stapled Peptides

Stapled peptides were produced by BioSynthesis (Lewisville, TX, USA) using Fmoc solid phase peptide synthesis. Peptides were stapled by ring-closing metathesis using Grubbs' First Generation Catalyst, and purified by preparative C18 reversed-phase HPLC. The purity of the peptides was confirmed as >95% by analytical HPLC and by MALDI-TOF MS.

Sample Preparation

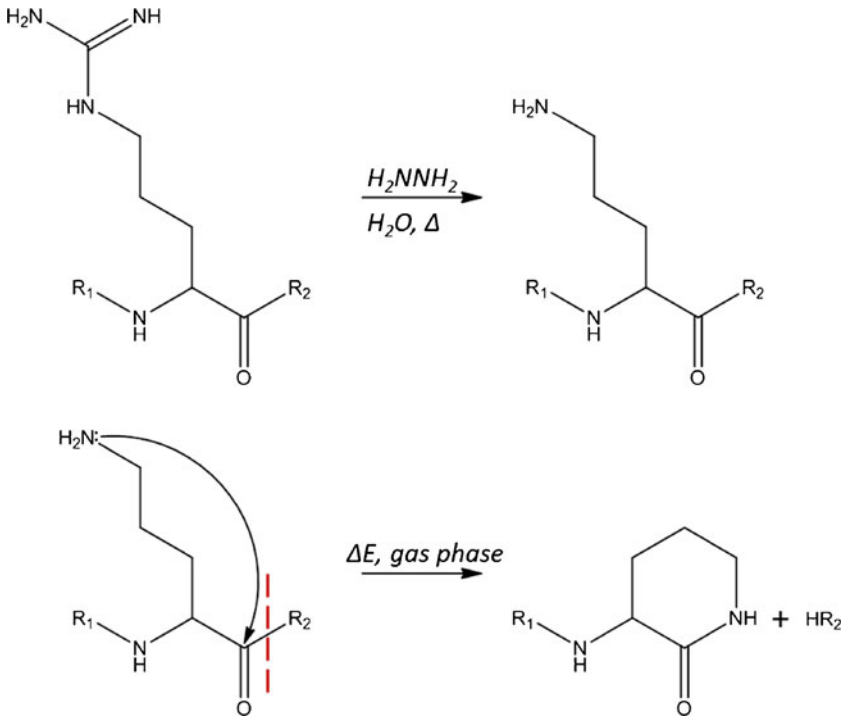
Unmodified samples were diluted to 5 uM with an equal mixture of methanol and water with 1% formic acid prior to direct infusion ESI. Stapled peptides and SFTI-1 were mixed with excess hydrazine hydrate for 4 h at 55 °C in water to promote the conversion of arginine to ornithine. The reaction mixture was dried under vacuum (Thermo Savant DNA 120 Speedvac Concentrator, San Jose, CA, USA) to remove organics and diluted to 5 uM with an equal mixture of methanol and water with 1% formic acid prior to direct infusion ESI. After the incubation of SFTI-1 with hydrazine hydrate, the intramolecular disulfide bond was reduced with dithiothreitol (100 mM in water) for 45 min at 55 °C and alkylated with iodoacetamide (100 mM in water) for 45 min protected from light at room temperature.

Mass Spectrometry and Photodissociation

All MS experiments were performed on a Thermo Scientific Orbitrap Elite mass spectrometer (San Jose, CA, USA) custom fit with an unfocused, non-collimated Coherent ExciStar 193 nm excimer laser (Santa Clara, CA, USA) to perform ultraviolet photodissociation, as previously described [46, 47]. Peptides, both modified and unmodified, were analyzed by direct infusion electrospray ionization with a spray voltage of 4 kV and a capillary temperature of 275 °C. For all CID experiments, the normalized collision energy (NCE) was varied to reduce the precursor to approximately 10%–15% relative abundance during an activation period of 10 ms (this is sufficient to provide rich fragmentation without excessive ejection of the precursor ion during resonance exCitation). For all UVPD experiments, a laser power of 1.5–2.5 mJ/pulse and one to three 5-ns pulses were used. The resulting MS/MS spectra were interpreted manually as well as by using a custom fragment ion prediction algorithm that was developed in-house.

Development of a Custom Algorithm for Assignment of Fragment Ions

Several algorithms have recently been developed to assist with the characterization of cyclic peptides. Two of the most recent algorithms are CYCLONE and CycloBranch,



Scheme 1. Conversion of an arginine residue into an ornithine residue results in a mass shift of 42 Da and proceeds in the presence of hydrazine. Upon gas-phase activation, the ornithine residue cyclizes via nucleophilic attack and the adjacent amide bond is heterolytically cleaved. This figure is adapted from Ref. [40]

which were developed to characterize cyclic peptides via a de novo strategy [48, 49]. An in-house algorithm was developed in C# to assign fragment ions of cyclic peptides based on interpretation of 193 nm UVPD mass spectra. Unlike CYCLONE and CycloBranch, this algorithm utilizes a naïve approach that calculates all

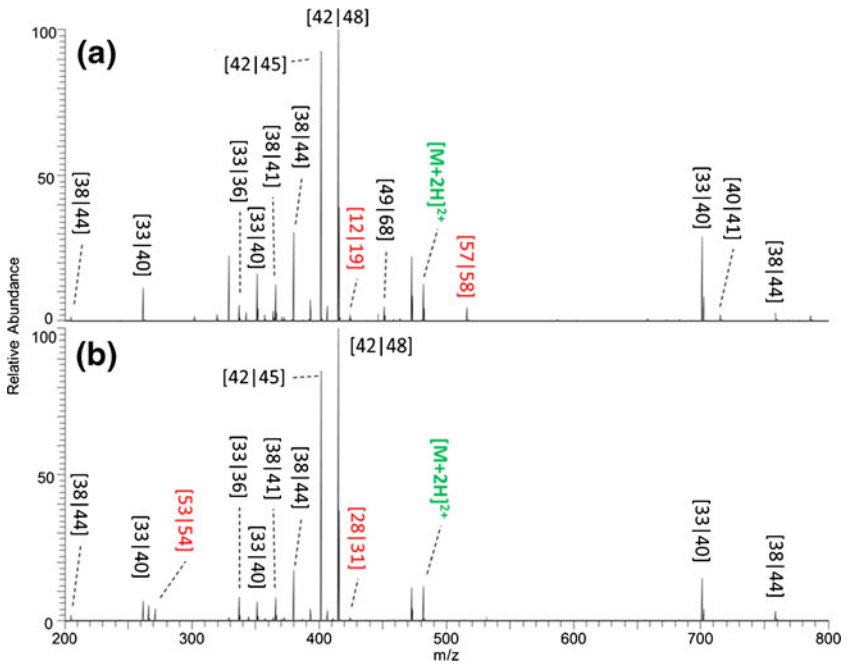


Figure 1. CID mass spectra (240 K resolution) of ornithine-containing stapled peptides. **(a)** [H_2N]-HG-X-AOA-X-GAD-[CO_2H]. **(b)** [H_2N]-HG-X-OAA-X-GAD-[CO_2H]. The "X" represents pentenyl alanine residues that have been stapled together through ruthenium catalyzed ring-closure metathesis. Red labels designate the unique fragment ions between the isobaric stapled peptides (regions around the fragments identified as [57|58] and [53|54] have been magnified 5× for clarity). See expanded regions of the spectra in Supplemental Figures S14 and S15 showing the differences in fragment ions between the two ornithine-containing stapled peptides

potential fragment ions which may arise from the cleavage of any bond in the peptide's structure and cross-ring cleavages based on candidate structures. Moreover, the custom algorithm accepts any type of candidate structure, including stapled peptides containing unnatural amino acids, unlike other available algorithms. The structures of the candidate peptides were generated in ChemDraw Perkin Elmer (Waltham, MA, USA). The structures were assigned atom numbers and implicit hydrogens were selected to be hidden. The final structure was saved as a cdxml file.

The XML from the cdxml files were then parsed by the algorithm and converted into an undirected graph (abstract data structure). A naïve approach was employed in which fragments were calculated through the systematic removal of each edge in the graph from the candidate molecule's structure. Additionally, with a ring size specified, fragments resulting from cross ring cleavages could also be calculated (resulting from the cleavage of two bonds). These calculated fragment ions were saved in a SQLite database [50], which could be exported into an Excel file. The ions in the MS/MS spectra were then manually compared with the theoretically calculated masses.

Results and Discussion

Conversion of arginine to ornithine by deguanidination in the presence of hydrazine causes a mass shift of 42 Da, as shown in Scheme 1. Subsequent activation of an ornithine-containing peptide may result in cyclization via nucleophilic attack of the side-chain amine of the ornithine residue on a neighboring carbonyl carbon, causing a heterolytic cleavage of the adjacent amide bond. This process is referred to as the "ornithine effect," and, in short, is based on a decrease in proton affinity in the gas phase when an arginine residue is converted to an ornithine residue (see reference [40] for a complete explanation of the ornithine effect and see reference [42], which reports the gas-phase proton affinity of ornithine). This site-specific cleavage affords a facile, predictable fragmentation pathway for arginine-containing peptides and offers a convenient way to convert cyclic peptides into acyclic ones. In this study, both CID and UVPD are implemented to induce the ornithine effect as a means to simplify the characterization of stapled and cyclic peptides.

When analyzing the MS/MS spectra of stapled and cyclic peptides, there is an inherent challenge in nomenclature

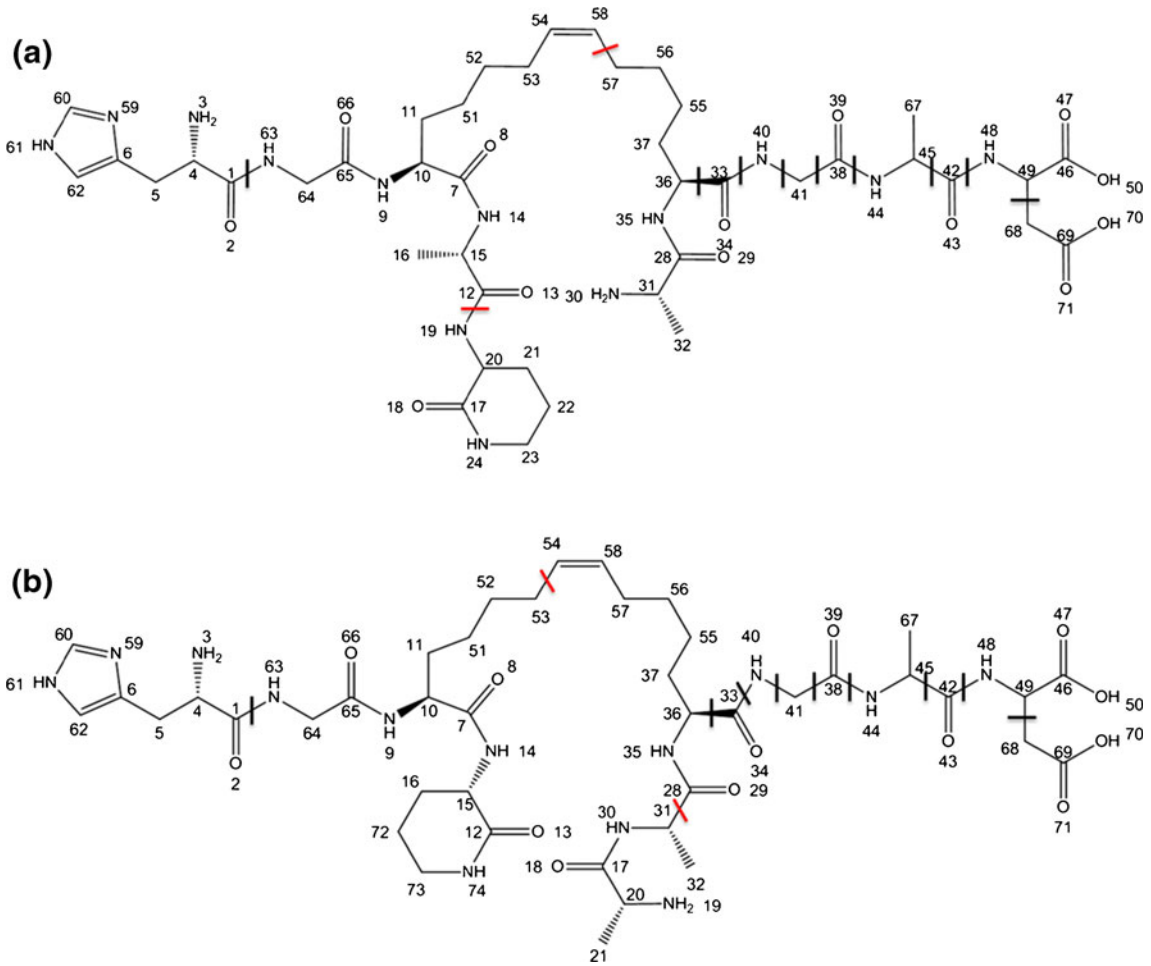


Figure 2. Fragmentation maps obtained using CID (240 K resolution) of ornithine-containing stapled peptides. **(a)** [H_2N]-HG-X-AOA-X-GAD-[CO_2H]. **(b)** [H_2N]-HG-X-OAA-X-GAD-[CO_2H]. The "X" represents pentenyl alanine residues that have been stapled together through ruthenium catalyzed ring-closure metathesis. Unique fragment ions detected for the ornithine-containing peptides are indicated in red

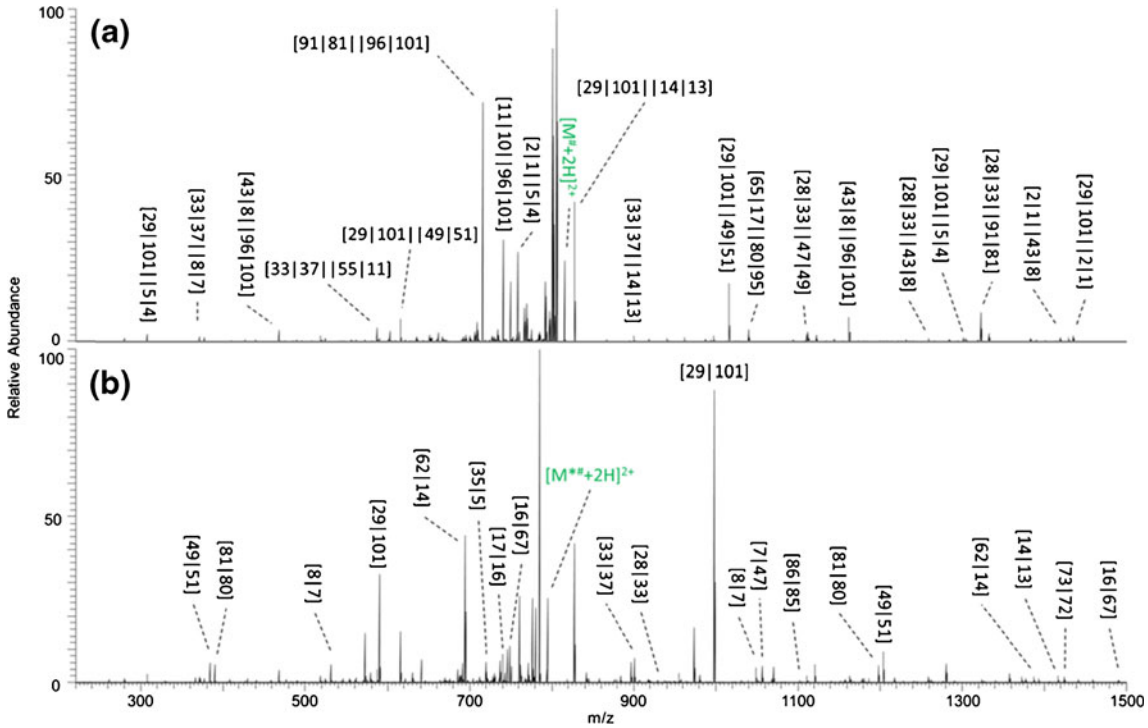


Figure 3. CID mass spectra (240 K resolution) of doubly charged **(a)** reduced and alkylated unmodified SFTI-1 (1629.90 Da) and **(b)** reduced and alkylated ornithine-modified SFTI-1 (1587.86 Da). (NCE = 24) An asterisk (*) represents presence of the modification from arginine to ornithine and a pound symbol (#) represents reduction and alkylation of the disulfide bond

associated with the fragment ions due to the presence of multiple branches or the lack of any terminal positions. In this study, an in-house algorithm was developed to assist in the identification and assignment of the resulting fragment ions.

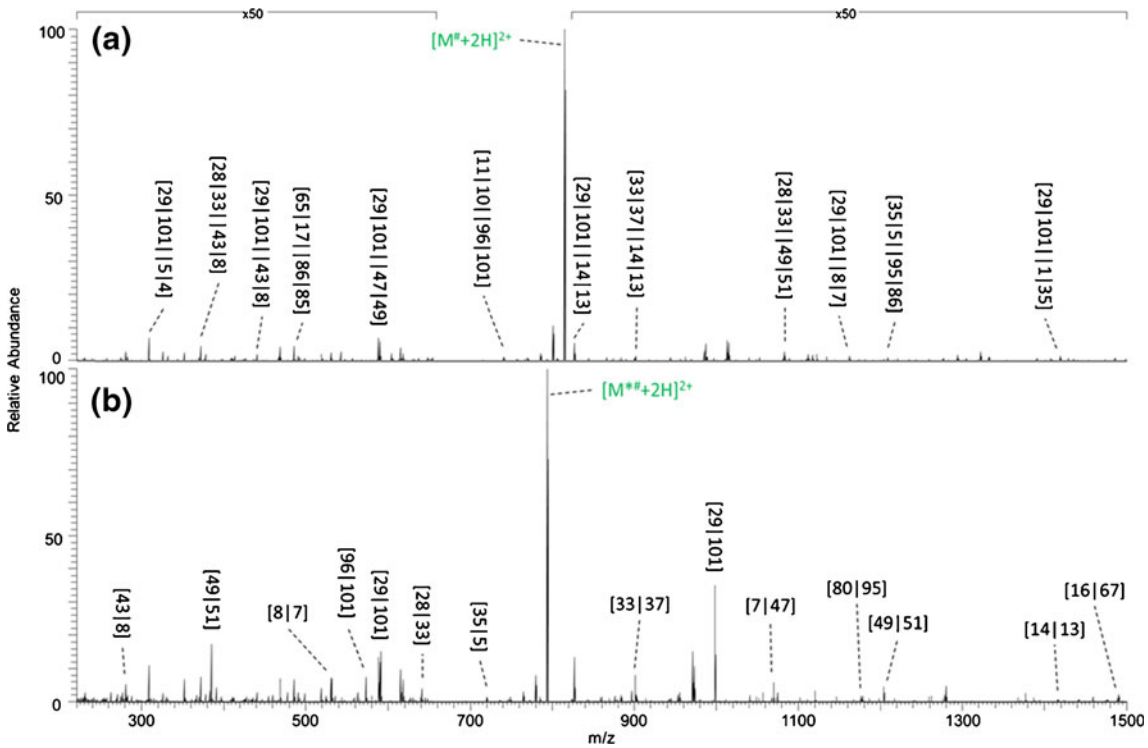


Figure 4. UVPD mass spectra (2.5 mJ, 1 pulse, 240 K resolution) of doubly charged **(a)** reduced and alkylated unmodified SFTI-1 (1629.90 Da) and **(b)** reduced and alkylated ornithine-modified SFTI-1 (1587.86 Da). An asterisk (*) represents presence of the modification from arginine to ornithine and a pound symbol (#) represents reduction and alkylation of the disulfide bond

For the stapled and cyclic peptides, a fragment ion involving the cyclic portion can only be produced via cleavages of two bonds (one in the cyclic region and one in the linear segment, typically creating an internal ion) or via a cross-ring cleavage. A single bond cleavage between atom numbers 1 and 2 is annotated as "[atom number 1 | atom number 2]" and the cleavage of two bonds between atom numbers 1 and 2 and atom numbers 3 and 4 simultaneously is annotated as "[atom number 1 | atom number 2 || atom number 3 | atom number 4]". If multiple cleavages lead to the same *m/z* ratio, all possible fragments are listed.

Two variants of a stapled peptide with sequences [H_2N]-HG-X-ARA-X-GAD-[CO_2H] and [H_2N]-HG-X-RAA-X-GAD-[CO_2H] (Supplementary Figure S1) were characterized. The "X" represents pentenyl alanine residues that have been stapled together through ruthenium-catalyzed ring-closure metathesis.The ESI mass spectra of one of the unmodified and ornithine-modified stapled peptides (Supplementary Figure S2) show that the conversion of the stapled peptide to the ornithine-containing analogue was an efficient reaction. As demonstrated in Supplementary Figure S3, the CID mass spectra of the two unmodified stapled peptides are nearly identical, resulting in no fragment ions that are unique to either of the two isomeric peptides (fragmentation maps for the unmodified stapled peptides are shown in Supplementary Figure S4). However, after conversion of the Arg residues to ornithine groups, unique fragments are identified for each (Figure 1a and b). Cleavages between atoms numbered 57 and 58 (annotated as [57|58]) and 12 and 19 ([12|19]) for [H_2N]-HG-X-AOA-X-GAD-[CO_2H] and cleavages between atoms numbered 53 and 54 ([53|54]) and atoms numbered 28 and 31 ([28|31]) for [H_2N]-HG-X-OAA-X-GAD-[CO_2H] are observed. Expanded regions of these key parts of the spectra are shown in Supplementary Figures S5 and S6. Fragmentation maps for each of the two modified stapled peptides are shown in Figure 2. The two unmodified isomeric peptides have no unique fragment ions in the MS/MS spectra, making the identification of location of the arginine residue within the stapled region impossible.

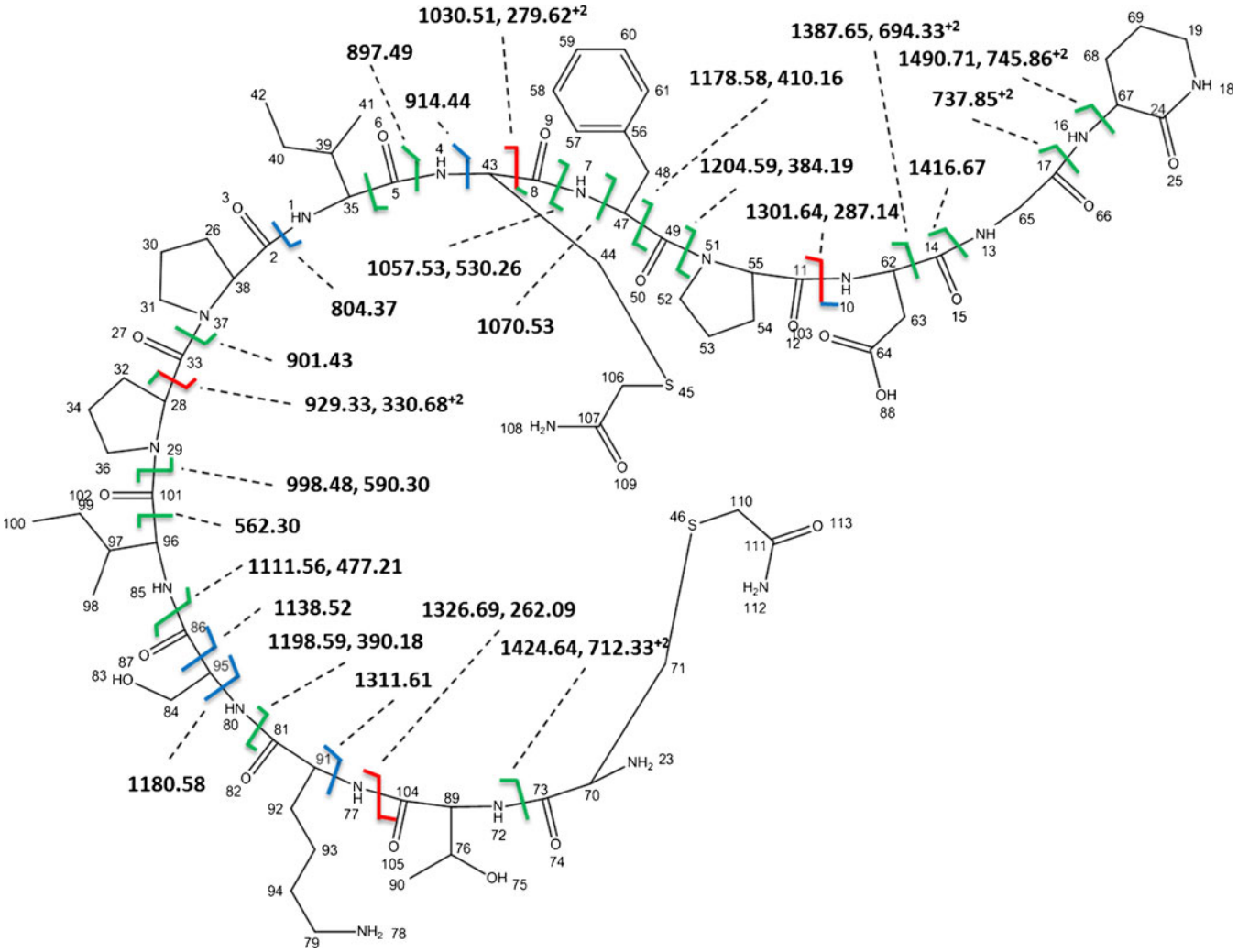


Figure 5. Fragmentation map of ornithine-modified SFTI-1 that has been reduced and alkylated. Red cleavages correspond to those fragment ions unique to CID; blue cleavages correspond to fragment ions unique to UVPD; green cleavages correspond to fragment ions produced by CID and UVPD

However, as shown in Figure 2a and b, there is clear indication that the ring opening and lactam formation arising from the ornithine effect lead to generation of unique product ions upon MS/MS. The unique ions of particular interest evolve from cleavages [12|19] and [57|58] for [H_2N]-HG-X-AOA-X-GAD-[CO_2H], and [28|31] and [53|54] for [H_2N]-HG-X-OAA-X-GAD-[CO_2H]. In addition to the CID spectra detailed here, the UVPD mass spectra and associated fragmentation maps are shown in Supplementary Figures S7 and S8. A few additional unique fragment ions are produced by UVPD of the ornithine-modified peptides relative to the unmodified peptides. As another approach, stapled peptides containing proteolytically recognized residues, such as Arg or Lys, can be subjected to proteolysis to cleave the cyclic stapled segment. The products generated upon tryptic digestion of the two stapled peptides are shown in Supplementary Figures S9 to S11. Tryptic digestion can result in more complex mixtures if multiple arginine and lysine residues are present.

Sunflower trypsin inhibitor-1 (SFTI-1) is another prime candidate to evaluate the utility of the ornithine effect for improving the characterization of cyclic peptides. Similar to the hurdle with analysis of stapled peptides, two bond cleavages are required to generate diagnostic fragment ions for cyclic peptides like SFTI-1. The use of the custom algorithm to assign *m/z* values for all possible cross-ring cleavage products is vital for predicting and assigning fragment ions for SFTI-1. SFTI-1 has a single arginine residue within its cyclic structure, which, upon conversion to an ornithine group and activation in the gas phase, causes a ring opening event that exposes a significant portion of the molecule (Supplementary Figure S12). The arginine residue converts readily to an ornithine residue in the presence of hydrazine hydrate, as shown in the ESI mass spectrum (Supplementary Figure S13). Furthermore, reduction and alkylation of the disulfide bond constraining the cyclic portion of the molecule was performed, allowing more extensive sequence coverage of the biomolecule. Upon CID (Figure 3) and UVPD (Figure 4), the greatest number of diagnostic ions are produced and identified for the ornithine-modified peptide after reduction and alkylation (Figures 3b and 4b). A complete list of the identified fragments and the associated *m/z* values are provided in Supplementary Table S11, and the most prominent ones for the ornithine-modified SFTI-1 after reduction and alkylation are shown in Figure 5. Additionally, CID and UVPD of unmodified SFTI-1 and ornithine-modified SFTI-1 without reduction and alkylation were performed and the resulting spectra are shown in Supplementary Figures S14 and S15, respectively. For comparison, a fragmentation map similar to Figure 5 is provided for the unmodified SFTI-1 (i.e., reduced and alkylated, but without the ornithine modification) in Supplementary Figure S16. Although there were still a number of fragment ions identified for unmodified SFTI-1 (reduced and alkylated but without the ornithine modification), the ambiguity associated with the cyclic nature of the peptide impeded the assignment of product ions. In essence, several of the fragment ions may arise from multiple fragmentation points across the cyclic peptide, hence leading to the redundancies in Supplementary Table S11. Owing to this ambiguity, exploiting the ornithine effect in tandem with reduction and alkylation leads to more confident characterization of the peptide.

As an alternative to the ornithinylation approach, tryptic proteolysis of SFTI-1 was undertaken. Tryptic digestion of SFTI-1, either with reduction/alkylation or without, failed to cause ring opening. Because SFTI-1 is a known trypsin inhibitor [51, 52], this result was not surprising and emphasizes the need for alternative approaches to facilitate the characterization of unusual peptides.

Conclusions

Conversion of arginine residues to ornithine residues provided an effective way to promote ring opening reactions of stapled or cyclic peptides chemically rather than enzymatically, with greater specificity than tryptic digestion. The preferential heterolytic cleavage C-terminal to the ornithine residue upon activation led to an N-terminal ion that terminated with a six-membered lactam ring. The ornithine effect allowed characterization of isomeric stapled peptides as well as the cyclic peptide SFTI-1 upon CID or UVPD. A custom algorithm was developed to facilitate assignment of mass values to all possible fragment ions created upon cleavage of two bonds, including cross-ring cleavages. Use of this algorithm simplified the interpretation and assignment of fragment ions produced from cyclic peptides.

Acknowledgments

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